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FIBRINOLYSIS

Enhancement of Recombinant Tissue Plasminogen Activator-induced Reperfusion by Recombinant Tick Anticoagulant Peptide, A Selective Factor Xa Inhibitor, in a Canine Model of Femoral Arterial Thrombosis

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SUMMARY. **Tick anticoagulant peptide (TAP) is a 60 amino acid protein originally isolated from the soft tick, *Ornithodoros moubata*, which exhibits potent anticoagulant properties due to its selective inhibition of blood coagulation factor Xa. We evaluated a recombinant version of TAP (rTAP) for its ability to accelerate recombinant tissue plasminogen activator (rt-PA)-mediated lysis of an occlusive thrombus and prevent acute reocclusion in a canine model of femoral artery thrombosis. An occlusive thrombus was formed by insertion of a thrombogenic copper coil into the femoral artery of anesthetized dogs. Blood flow velocity was monitored directly and continuously by Doppler flowmetry. 60 min after occlusion, dogs received an i.v. infusion of either saline or rTAP (0.5, 2.5 or 8.0 μg/kg/min), followed 45 min later by rt-PA (0.8 mg/kg, i.v. over 90 min; n=8/group). The saline and rTAP infusions were discontinued 1 h after stopping the rt-PA. All dogs achieved reperfusion, with a time to reperfusion in the saline-treated (vehicle) group (administered rt-PA alone) of 61±7 min. The time to reperfusion was slightly decreased in the 0.5 ug/kg/min rTAP group (47±4 min, p=NS). In the groups administered rTAP at 2.5 and 8.0 ug/kg/min, significant reductions in the time to reperfusion were observed (28±4 and 32±5 mins, respectively, p<0.05). Following termination of the rt-PA, all vehicle dogs reoccluded in 37±11 min. The lowest dose of rTAP, 0.5 ug/kg/min, had no effect on either the reocclusion incidence or time (8/8 in 39±7 min). In contrast, the two higher doses of rTAP maintained vessel patency in all dogs during the rTAP infusion period and dramatically delayed the time to reocclusion. However, 7/8 dogs eventually reoccluded in 117±12 and 140±9 min for the groups receiving rTAP infusions of 2.5 and 8.0 ug/kg/min, respectively. Maximal elevations in activated partial thromboplastin time or template bleeding time associated with the rTAP administration were only 1.3- and 1.1-fold of baseline values, respectively. The dramatic effect of factor Xa inhibition on the efficacy of rt-PA-mediated reperfusion and acute reocclusion following rt-PA suggests that factor Xa inhibition may represent a potentially useful therapeutic adjunct to thrombolytic therapy.**

INTRODUCTION

The therapeutic benefit of thrombolytic agents is dependent upon rapid and sustained recanalization after occlusive thrombus formation.[1,2] Despite proven efficacy in achieving recanalization, persistant occlusion and unpredictable acute reocclusion, which occurs in a high percentage of patients receiving thrombolytic therapy, continue to be major limitations associated with this procedure.[3–5] Although anticoagulant and antiplatelet agents are presently administered to enhance reperfusion and prevent reocclusion, their efficacy has not been uniformly effective.[6–9]

Following damage to the vascular endothelium, platelet adhesion, activation and aggregation occur. Activation of the coagulation cascade results in the formation of the serine protease factor Xa. Factor Xa, in combination with the non-enzymatic cofactor Va and calcium, assembles into the catalytic prothrombinase complex on the surface of adhered, activated platelets resulting in the formation of thrombin from prothrombin within the developing thrombus.[10] In addition to its potent direct proaggregatory effect on platelets, thrombin catalyzes the generation of fibrin from fibrinogen and the activation of factor XIII (XIIIa), resulting in the continued growth and stabilization of the evolving thrombus.[11,12] Thrombin also promotes its own formation and potentiates the above reactions through

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the positive feedback activation of factors VIII, V and XI.[13] The pivotal role of thrombin in arterial thrombus formation has lead to the development of several direct and highly selective inhibitors of this enzyme.[14–19] Although direct thrombin inhibitors have demonstrated antithrombotic efficacy in several animal models of arterial thrombosis, they have been associated with impairment of normal hemostasis as evidenced by the prolongation of bleeding time and/or coagulation time.

The penultimate position of factor Xa in the coagulation cascade makes it an attractive target for pharmacological intervention. Tick anticoagulant peptide (TAP) is a 60 amino acid protein initially isolated from the soft tick, *Ornithodoros moubata*, which exhibits potent anticoagulant properties due to the stoichiometric and highly selective inhibition of factor Xa.[20,21] In this study we evaluated the effects of a recombinant version of TAP (rTAP)[22,23] on recombinant tissue plasminogen activator (rt-PA)-mediated reperfusion and acute reocclusion in a canine model of femoral arterial thrombosis. The results demonstrate that rTAP significantly accelerated reperfusion and delayed acute reocclusion without impairment of primary hemostasis.

MATERIALS AND METHODS

Surgical Preparation

32 adult mongrel dogs of either sex weighing 8 to 13 kg were used in this non-survival study. The dogs were anesthetized with sodium pentobarbital (35 mg/kg, i.v.), intubated and ventilated with room air through a Harvard respirator. Blood pO_2, pCO_2 and pH were continuously monitored and adjusted to physiological levels, as needed. Surgical preparation began with the isolation of the left carotid artery and jugular vein. The left jugular vein was cannulated with a double lumen catheter in order to administer drugs separately. The right brachial artery was cannulated with a Tygon catheter to measure arterial blood pressure. Both femoral arteries were isolated, leaving intact the most prominent muscular side branch. A cuff-type Doppler flow probe was placed on both femoral arteries just distal to the side branch. Mean and phasic femoral artery blood flow velocity (FABFV) were measured with a pulsed Doppler flowmeter (Hartley, Houston, TX). Arterial blood pressure, heart rate and FABFV were recorded continuously on a Hewlett Packard physiological recorder.

Induction of Arterial Thrombi

Following a stabilization period of 30 min, an arterial thrombus was formed by the placement of a thrombogenic copper coil into either femoral artery, as described previously.[19,24] A shortened 8F polyurethane pigtail catheter (USCI, Billerica, MA) was inserted into the left carotid artery and advanced to either femoral artery. A teflon-coated guidewire (Cook Co., Bloomington, IN) was then passed through the hollow catheter and extended several centimeters beyond the end of the catheter. The hollow catheter was then removed while the guidewire was held in place. A coil (8.0 mm long), equal in diameter to the outside diameter of the femoral artery (as measured by calipers: range=2.3 to 2.8 mm), was placed over the guidewire and advanced by the catheter to the femoral artery where it was secured 6.0 mm distal to the intact muscular side branch. The guidewire was removed and 2–3 ml saline were flushed down through the catheter and femoral segment. The Doppler flow probe was repositioned proximal to the copper coil and just distal to the intact side branch.

Experimental Protocol

Dogs were assigned randomly to one of four treatment groups (Fig. 1). There were 8 dogs in each treatment group. All drugs were administered intravenously. One group received a continuous infusion of saline, 0.1 ml/min, and three additional groups received rTAP as a continuous infusion of either 0.5, 2.5 or 8.0 ug/kg/min. Drug administration was initiated 1 h after occlusive thrombus formation and maintained until 1 h after terminating the rt-PA infusion for a total infusion time of 195 min. 45 min after starting the drug administration (105 min after thrombus formation), all dogs received a continuous infusion of rt-PA, total dose=0.8 mg/kg over 90 min, i.v. (Activase, Genentech, South San Francisco, CA, specific activity=580000 IU/mg). An additional 120-min observation period extended beyond the termination of the drug infusions. The coil and thrombus were removed at the end of the experimental period (375 min after thrombus formation) and wet thrombus mass was determined. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee and complied with Federal Regulations.

Drugs and Solutions

All drugs were prepared on the day of the experiment. rTAP was purified to homogeneity from a culture media of *Saccharomyces cerevisiae* as previously described.[22] All experiments were done from a single lot of rTAP dissolved in saline.

Hematological Evaluations

Four ml of arterial blood were withdrawn into a plastic syringe containing 0.4 ml of 3.8% trisodium citrate solution. The blood was centrifuged at 5°C for 10 min at 2000×g. The plasma was removed and

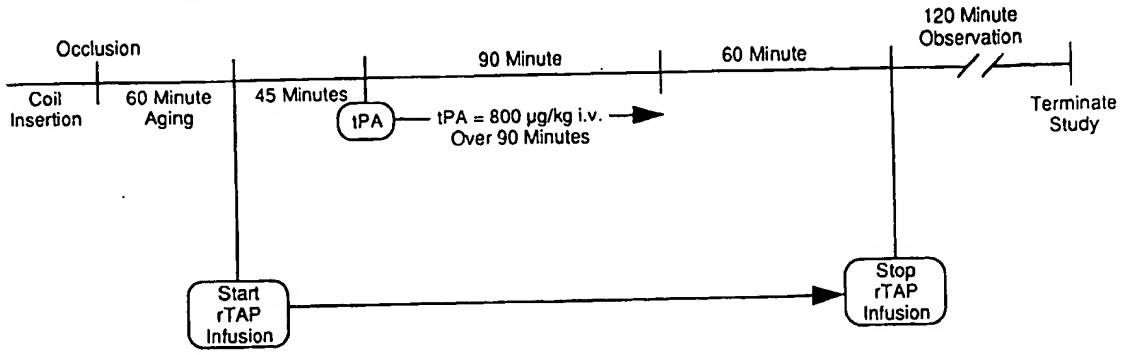

Fig. 1 Experimental protocol with conjunctive treatment groups.

stored on ice for immediate assay or frozen at −70°C. The activated partial thromboplastin time (APTT) was determined with an automated APTT kit (Organon Teknika Corp., Durham, NC). Buccal mucosa bleeding times (BT) were measured with a Simplate bleeding time device (Organon Teknika Corp., Durham, NC), as previously described.[25] Uniform incisions were made with the Simplate on the mucous membrane of the upper lip of the dog, and the duration of bleeding was timed.

Plasma r-Tick Antiplatelet Peptide Concentrations

Plasma levels of rTAP were measured as described previously[22] in an assay utilizing purified human factor Xa and the chromogenic substrate Spectrozyme Xa (American Diagnostica, Greenwich, CT).

Criteria for Reperfusion and Reocclusion

The time to reperfusion was defined as the re-establishment of FABFV to at least 50% of control 'coil' FABFV (FABFV after placement of the coil) for a period of 5 min or any measureable continuous flow for 15 min after starting the r-tPA infusion. The time to reocclusion was defined as the first incidence of FABFV returning to zero after stopping the rt-PA infusion.

Statistical Analysis

Results are expressed as the mean±SEM. Within group comparisons were performed using an analysis of variance, followed by a Dunnett's test for multiple comparisons. Among group comparisons were performed using an analysis of variance, followed by a Neuman Keuls test. In all cases, $p<0.05$ was considered significant.

RESULTS

Arterial Reperfusion and Reocclusion

Following insertion of the copper coil, an occlusive thrombus was formed in all dogs in all groups in 12±1 min as indicated by a progressive decline of FABFV to zero. Control FABFV was measured immediately after coil placement and was not significantly different among any of the groups. Systemic infusion of rt-PA restored arterial blood flow in all dogs. In the saline-treated group (vehicle), administered rt-PA alone, the time to reperfusion was 61±7 min (Table 1). The time to reperfusion was slightly decreased in the group administered rTAP at a dose of 0.5 ug/kg/min, (47±4 min, p=NS). In contrast, the time to reperfusion was significantly reduced in the groups that received rTAP at doses of either 2.5 or 8.0 ug/kg/min, (28±4 and 32±5 min, respectively, $p<0.05$).

The time to reocclusion was defined as the first incidence of FABFV returning to zero after terminating the infusion of rt-PA. The time to reocclusion for the vehicle group was 37±11 min, with 6 of 8 dogs reoccluding during the 60 min saline infusion following the termination of the rt-PA (Table 1). The two remaining dogs reoccluded within 28 min after termination of the saline infusion. During the infusion of rTAP at 0.5 ug/kg/min, 6 of 8 dogs reoccluded, with 2 animals remaining patent until 10 min after the termination of the rTAP infusion. There was no improvement in the time to reocclusion in the 0.5 ug/kg/min rTAP group compared to the vehicle group.

Table 1 Reperfusion and reocclusion in a canine model of femoral arterial thrombosis

Group	Incidence of reperfusion	Time to reperfusion[a] (min)	Incidence of reocclusion	Time to reocclusion[a,b] (min)	Thrombus mass[a] (mg)
Vehicle	8/8	61±7	8/8	37±11	38±5
rTAP 0·6μg/kg/min	8/8	47±4	8/8	39±7	34±4
rTAP 2.5μg/kg/min	8/8	28±4[c]	7/8	117±12[b,c]	33±5
rTAP 8.0μg/kg/min	8/8	32±5[c]	7/8	140±9[b,c]	21±4[c]

[a] Values shown are mean±SEM.
[b] Mean time to reocclusion excludes one preparation in each group which failed to reocclude during the experimental protocol.
[c] $p<0.05$ compared to vehicle.

(39±7 vs 37±11 min, respectively). In contrast to the 0.5 ug/kg/min group, there was no reocclusion in any dogs receiving rTAP at 2.5 or 8.0 ug/kg/min during the infusion of the inhibitor. All but one dog in each group reoccluded before the end of the experimental protocol after terminating the 2.5 and 8.0 ug/kg/min rTAP infusions. However, the times to reocclusion were significantly delayed in those preparations in the 2.5 and 8.0 ug/kg/min rTAP groups which did reocclude (117±12 and 140±9 min, respectively, $p<0.05$) compared to the vehicle group (37±11 min). Thrombus mass was significantly reduced following the administration of the 8.0 ug/kg/min rTAP dose (Table 1).

Femoral Arterial Blood Flow Restoration

The extent and duration of reperfusion following thrombolysis, measured as integrated FABFV profiles, are shown in Figure 2. Following initial reperfusion, FABFV gradually increased and was maintained in all dogs during the 90 min rt-PA infusion. The maximal extent of FABFV achieved for the vehicle and rTAP doses of 0.5, 2.5 and 8.0 ug/kg/min were 91±6%, 99±1%, 83±7% and 88±12% of baseline values, respectively. Following termination of the rt-PA infusion, FABFV rapidly declined in the vehicle and 0.5 ug/kg/min rTAP-treated groups. In contrast, FABFV was maintained throughout the 60 min rTAP infusion in all dogs administered 2.5 and 8.0 ug/kg/min rTAP. Following termination of the rTAP infusions at these doses, flow gradually returned toward zero reflecting a significant delay in and, for one preparation in each group, a prevention of reocclusion.

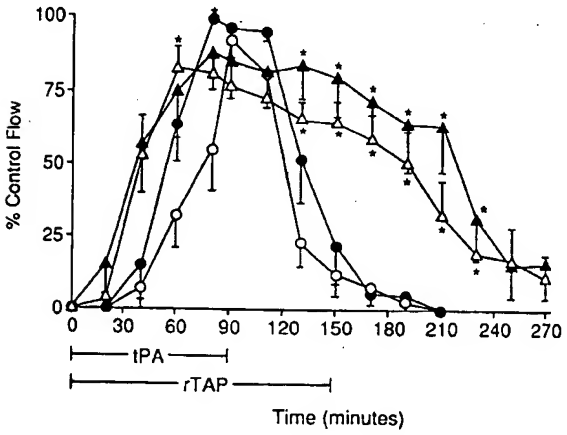

Fig. 2 Effects of rTAP on FABFV. Restoration of FABFV during and after rt-PA-induced thrombolysis, for those groups receiving either vehicle (○) or rTAP, 0.5 μg/kg/min (●), 2.5 μg/kg/min (△) or 8.0 μg/kg/min (▲). 0 min=start of rt-PA infusion. The periods spanning the rt-PA and rTAP infusions are indicated in brackets. Values are represented as the mean±SEM. * $p<0.05$ vs. vehicle.

Ex Vivo Clotting Assay and Bleeding Times

Negligible elevations in APTT values (<1.2-fold of baseline values) were observed in the vehicle group (Table 2). Similar elevations in APTT were also observed in the three rTAP groups. Only insignificant elevations in BT were observed in any of the treatment groups (Table 2).

Plasma r-Tick Antiplatelet Peptide Concentrations

Plasma rTAP concentrations for the 2.5 and 8.0 ug/kg/min doses were determined at 60, 120 and 180 min after starting the rTAP administration and 45 min after stopping the infusion (Fig. 3). Plasma concentrations increased to maximum levels of 87.8±3.1 and 282.5±23.1 nM at 180 min after the start of rTAP administration for the 2.5 and 8.0 ug/kg/min doses, respectively. Within 45 min after stopping the infusions, the plasma concentrations decreased to 34.6±2.7 and 72.8±5.8 nM for the 2.5 and 8.0 ug/kg/min doses, respectively.

Hemodynamics

Changes in mean arterial blood pressure (MAP) and heart rate (HR) throughout the experimental period are shown in Table 3. Dogs in all groups had similar

Table 2 Activated partial thromboplastin times (APTT) and bleeding times (BT)

Group	Minutes after initiating rTAP administration 0	60	120	180	240
Vehicle					
APTT	11.2±0.3	10.9±0.3	13.3±1.4	11.3±0.3	10.9±0.4
BT	2.2±0.3	2.1±0.2	2.2±0.2	2.3±0.3	2.1±0.2
rTAP 0.5 μg/kg/min					
APTT	10.0±0.3	11.0±0.7	12.9±1.4	10.5±0.5	10.5±0.4
BT	1.7±0.8	1.8±0.2	1.9±0.1	1.9±0.2	1.6±0.2
rTAP 2.5 μg/kg/min					
APTT	9.9±0.1	10.3±0.2	10.3±0.1	10.2±0.2	9.9±0.2
BT	2.5±0.2	2.8±0.3	3.0±0.4	2.8±0.4	2.8±0.3
rTAP 8.0 μg/kg/min					
APTT	10.5±0.3	11.0±0.1	11.0±0.2	10.9±0.3	10.4±0.2
BT	2.6±0.2	2.9±0.3	2.5±0.2	2.7±0.2	2.5±0.2

APTT=activated partial thromboplastin time (sec); BT=bleeding time (min)
0–195 min=rTAP infusion; 45–135 min=tPA infusion (0.8 mg/kg, total dose)
Values shown are mean±SEM.

control values for MAP and HR. Moderate decreases in MAP and elevations in HR were observed in the groups receiving either 2.5 or 8.0 ug/kg/min rTAP at the end of the experimental protocol, well after the termination of the rTAP infusions.

DISCUSSION AND CONCLUSIONS

Tick anticoagulant peptide is a 60 amino acid protein that was originally isolated from the soft tick, *Ornithodorus moubata* and subsequently produced as a recombinant protein (rTAP) in yeast.[22,23] rTAP is a highly selective and potent inhibitor of blood coagulation factor Xa.[20,21] rTAP has previously been shown to prevent venous thrombus formation in rabbits, suppress systemic elevations of fibrinopeptide A in a rhesus monkey model of mild disseminated intravascular coagulation and prevent thrombus formation in a primate model of high-shear, platelet dependent arterial thrombosis.[22,26,27] In addition, rTAP has been shown to enhance thrombolysis and prevent reocclusion in a canine model of acute coronary thrombosis with a superimposed critical stenosis.[28] In the present study, we evaluated rTAP for its ability to accelerate rt-PA-mediated lysis of an occlusive thrombus and prevent or delay acute reocclusion in a canine model of femoral arterial thrombosis. In this canine model, an occlusive thrombus is formed by insertion of a thrombogenic copper coil into the femoral artery. The thrombus that forms has been previously shown to be predominantly composed of platelets and fibrin, with platelets constituting the greatest fraction of the thrombus.[24] The mechanisms of initial thrombus formation and reocclusion are predominantly thrombotic in nature. Agents with vasodilatory properties have not demonstrated antithrombotic efficacy, whereas direct thrombin inhibition prevented initial thrombus formation and rethrombosis after lysis.[19] The results of this study demonstrate that rTAP accelerated rt-PA-induced reperfusion and delayed acute reocclusion without impairment of primary hemostasis as reflected by modest elevations in the APTT and bleeding times.

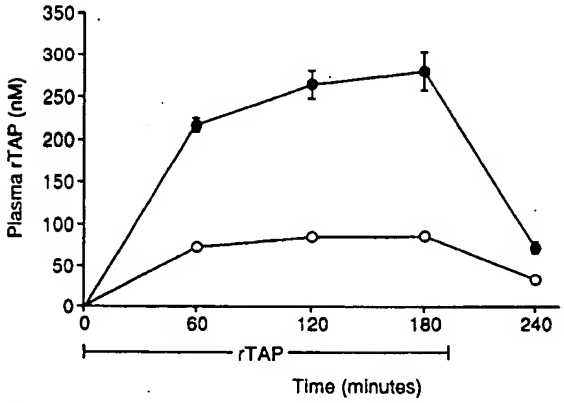

Fig. 3 Plasma rTAP concentrations (nM) for the 2.5 (O) and 8.0 (●) μg/kg/min dosage groups. The rTAP was administered as a continuous i.v. infusion from 0–195 min and the plasma levels of rTAP were determined at 60, 120 and 180 min after initiating the rTAP infusions and at 45 min after stopping the infusions.

The importance of thrombin as a primary mediator of arterial thrombus formation has been demonstrated in several animal models by the antithrombotic efficacy of direct (antithrombin III-independent) inhibitors of thrombin.[14–19] The results of the present study extend our knowledge of this process in which thrombin generation is a critical component of arterial thrombogenesis. By inhibiting factor Xa within the prothrombinase complex, rTAP effectively blocks

Table 3 Hemodynamic data

Group	Minutes after thrombus formation 0	60	105	195	255	375
Vehicle						
MAP	132±5	128±6	125±5	127±6	124±5	118±5
HR	149±9	151±8	146±9	152±8	151±10	162±11
rTAP 0.5 μg/kg/min						
MAP	141±4	134±4	141±3	141±3	138±3	131±2
HR	169±10	167±3	165±9	170±10	169±9	168±8
rTAP 2.5 μg/kg/min						
MAP	131±6	127±6	127±6	125±7	121±6	108±4[a]
HR	165±9	176±4	187±6	176±5	189±5[a]	192±6[a]
rTAP 8.0 μg/kg/min						
MAP	131±3	130±4	126±4	119±4	113±5[a]	104±4[a]
HR	150±11	153±9	164±9	163±8	172±10	181±8

MAP=mean arterial pressure (mmHg); HR=heart rate (beats/min)
60–255 min=rTAP infusion; 105–195 min=tPA infusion (0.8 mg/kg, total dose)
Values shown are mean±SEM.
[a] $p<0.05$ compared to 0 min.

thrombin formation at the site of the developing thrombus. The results with rTAP are supported by the findings of recent animal studies which evaluated agents that control the generation of thrombin by other regulatory mechanisms. Recombinant activated protein C, which inactivates the non-enzymatic cofactors Va and VIIIa, effectively suppressed systemic elevations of fibrinopeptide A levels and inhibited thrombus formation in a primate model of arterial thrombosis.[29] In addition, inhibition of tissue factor-induced coagulation mediated by the extrinsic pathway (tissue factor pathway inhibitor (TFPI)) or a modified factor IXa assembly into a catalytically inactive tenase complex (glutamyl-glycyl-arginyl-Factor IXa) have also demonstrated antithrombotic efficacy in animal models of arterial thrombosis.[30,31] These studies, combined with those utilizing direct thrombin inhibitors,[14–19] clearly demonstrate that arterial thrombogenesis can be effectively controlled by either the inhibition of thrombin generation or the direct inhibition of thrombin.

rTAP, administered as a continuous infusion of 2.5 ug/kg/min, significantly reduced the time to rt-PA-induced reperfusion and inhibited acute reocclusion in 100% of the dogs during the rTAP infusion. An accelerated time to reperfusion probably reflects the ability of rTAP to limit clot extension by interrupting thrombin-mediated fibrin formation and platelet deposition. In addition, thrombin activates factor XIII (XIIIa), which catalyzes the covalent crosslinking of fibrin.[12] Therefore, inhibition of thrombin or thrombin generation would prevent factor XIIIa-mediated crosslinking of fibrin strands as well as inhibitors of fibrinolysis such as alpha-2 antiplasmin thereby rendering the thrombus more susceptible to lysis.[32,33] In the clinical setting, the incidence of reocclusion after successful thrombolysis (reported as high as 15%) imposes an unpredictable limitation on the therapeutic benefit of thrombolytic therapy.[3–5] The primary mediator of thrombotic reocclusion may be ongoing platelet activation due to the residual thrombogenicity in the region of the damaged vessel. This hypothesis is supported by biochemical evidence that indicates that platelet activation continues to occur after reperfusion and the results of several animal studies that have demonstrated the antithrombotic efficacy of inhibitors directed against the IIb/IIIa platelet receptor.[34–38] The primary agonist of this continued platelet activation may be thrombin; the source being the reexposure of active clot-bound thrombin or the generation of new thrombin as a result of sustained prothrombinase activity. However, the inability of heparin to prevent reocclusion in our model of femoral artery thrombosis as well as several other animal models of arterial thrombosis, despite a marked anticoagulant effect, does not support a role for thrombin.[28,39–42] The ineffectiveness of heparin may be due to the weak activity of the heparin-antithrombin III complex to neutralize clot-bound thrombin associated with fibrin or factor Xa assembled in the prothrombinase complex.[43–45] Heparin may also be neutralized by several endogenous inhibitors released from activated platelets such as platelet factor 4.[46] The important role of thrombin as a mediator of reocclusion is also supported by the inability of aspirin to prevent acute reocclusion in our model of femoral artery thrombosis and the lack of effect of rTAP to inhibit ex vivo platelet aggregation to ADP and collagen.[28,39]

The antithrombotic efficacy of direct thrombin inhibitors and agents which block the platelet glycoprotein IIb/IIIa complex in several animal models has been associated with an impairment of normal hemostasis as reflected by significant elevations in

bleeding time.[35–38] In contrast, a fully antithrombotic dose of rTAP produced only a 1.2-fold elevation in template bleeding time. A similar uncoupling of antithrombotic efficacy and bleeding has been demonstrated in animal studies which have evaluated other agents that inhibit the generation of thrombin.[30,31,39] In addition, rTAP at a dose that accelerated the time to reperfusion and delayed reocclusion produced only a 1.1-fold elevation of APTT. However, this correlation between the ex vivo APTT and the antithrombotic efficacy of rTAP may be related to the nature of the clotting assay and the relative kinetic rates of association of this inhibitor with factor Xa.[47]

In conclusion, we have demonstrated that the potent and selective inhibition of factor Xa by rTAP can significantly accelerate rt-PA-induced thrombolysis and delay reocclusion without impairing primary hemostasis in a canine model of femoral artery thrombosis. The potent antithrombotic efficacy of rTAP in this model suggests that specific factor Xa inhibition may represent a pharmacologically useful approach to conjunctive thrombolysis.

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